

Original article

Effect of p21Ras *in vitro* intervention on the phenotype of airway smooth muscle cells from asthmatic rats

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Keywords: *asthma; p21Ras; phenotype*

Background An important physiological feature of asthma is the phenotypic change of airway smooth muscle cells (ASMCs), but the precise mechanisms behind the ASMCs' change remains unknown. Our study assessed whether p21Ras can directly modulate the phenotype of ASMCs.

Methods Rat ASMCs were treated with FTP III, a highly specific p21Ras inhibitor. ASMCs were identified via immunocytochemistry. The ultrastructure of cells was observed by electron microscopy, and the expression of α -actin was evaluated by Western blotting analysis. The levels of IL-6 and RANTES were measured by enzyme linked immunosorbent assay (ELISA).

Results It was observed that ASMCs in asthma exhibited a proliferative/secretory phenotype and were larger, denser and had many pseudopods, as well as increased signs of secretory organelles. Additionally, the level of α -actin, a marker of ASMCs, was reduced in asthmatic ASMCs and the secretion of IL-6 and RANTES was increased. When FTP III was added to asthmatic ASMCs it induced a contractile phenotype, with increased α -actin levels and reduced secretion of IL-6 and RANTES.

Conclusions It appears that p21Ras induces asthmatic ASMCs to a proliferative/secretory phenotype, but its inhibitor FTP III, can significantly reverse this phenotype. The role of p21Ras in the ASMCs may be a new target for asthma treatment.

Chin Med J 2011;124(1):32-37

METHODS

Asthma is an inflammatory airways disease typically characterized by intermittent chronic airway inflammation, airway remodeling, and airway hyper-responsiveness (AHR).¹ Airway smooth muscle cells (ASMCs) are a "super-cell" due to their multi-functional capacity in asthma, which may be due to its "plastic" nature to change phenotype. Phenotype change underpins the ability of ASMCs to contribute to acute bronchospasm, AHR, and airway remodeling. In addition, it also leads to the synthesis and secretion of inflammatory mediators.²⁻⁴ Furthermore, different ASMCs phenotypes may influence the expression of adhesion molecules on inflammatory cells, which would affect the airway inflammation in asthma.^{5,6} If the mechanism of phenotype change could be completely understood, it would appear to hold significant promise in identifying new targets for treatment of asthma.⁷

p21Ras, a guanine nucleotide-binding protein, is involved in a number of diverse biologic processes, including cell proliferation, differentiation, and apoptosis. Ras signaling is involved in a complex array of signaling pathways, where cross-talk, feedback loops, branch points and multi-component signaling complexes are recurring themes. Recent evidence suggests that p21ras acts as a point of convergence for diverse extracellular signal-stimulated pathways in ASM cells.^{8,9} It is well known that ASMCs play an important role in asthma and we want to assess whether p21Ras can be a treatment target in asthma by its modulation in ASMCs.

Animal sensitization and allergen exposure

Twenty male Sprague-Dawley rats, 4–6 weeks old and weighing from 150 to 180 g, were obtained from the Southern Medical University Animal Centre in Guangzhou, China. The rats were randomly divided into two groups, which included the asthma group and the control group. Each group had 10 rats. Rats were sensitized to become asthmatic as previously described.¹⁰ All experiments were approved by the Institutional Review Board and followed internationally accepted standard veterinary procedures.

Cultivation of rat airway smooth muscle cells

ASMCs were isolated following a modified protocol which was based on the method previously reported.¹¹ Briefly the trachea and lungs were completely isolated from sacrificed rats and then the extraneous membranes

DOI: 10.3760/cma.j.issn.0366-6999.2011.01.007

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This work was supported by the grant from the Guangdong Medical Research Foundation of China (No. A2005612).

and connective tissues were carefully dissected away, the trachea and bronchus were cut off, and the outer membrane scraped. The remaining tissue was cut into blocks with a volume about 1 mm×3 mm, and the blocks were digested for 30 minutes with 0.7–1.0 ml of 0.2% type I collagenase (Invitrogen, Carlsbad, CA, USA) in 25 ml tissue culture flasks. DMEM (Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS) (Gibco) was added and the cultures were incubated in a CO₂ incubator at 37°C. After 5–7 days the cells were 80% confluent. The cells were then removed with trypsin and transferred to a new flask where they were allowed to adhere for 30 minutes. The remaining media containing the non-adherent cells and debris was then removed. After this purification step, the desired cell purity had been obtained and third passage cells were used for experiments. The cells were identified as ASMCs by morphology and immunofluorescence staining.

Third passage cells from the control group were divided into the following four groups: (1) blank control group without any intervention agent; (2) induced control group treated with a stimulant mixture, 10 ng/ml each of IL- β , TNF- α , and IFN- γ (Preprotech, Rocky Hill, NJ, USA); (3) interventional control group I treated with 10 μ mol/L FPT III, a p21Ras inhibitor (Calbiochem, San Diego, CA, USA); (4) interventional control group II treated with both the stimulant mixture and 10 μ mol/L FPT III. The asthma group cells were also divided into four groups: (1) blank asthma group; (2) induced asthma group; (3) interventional asthma group I; (4) interventional asthma group II. Each of the eight groups was cultured in DMEM supplemented with 0.1% FCS for 24 hours, then cultured for 24 hours with the addition of their respective treatments. Supernatants or cells were then collected for analysis.

Transmission electron microscopy

Cells were released from the flasks by digestion with 0.25% trypsin then transferred to a centrifuge tube, centrifuged at 2000 r/min for 5 minutes, the supernatant discarded, the cell pellet was transferred to a 1.5 ml eppendorf tube, centrifuged, and fixed in 2.5% glutaraldehyde at 4°C. After fixation the material was washed and post-fixed, dehydrated, embedded, sectioned, stained, and finally observed under an electron microscope.

Western blotting

Proteins were extracted from cells with sodium dodecylsulfate (SDS) sample buffer, then subjected to 12% SDS-PAGE and transferred onto PVDF membranes (Pierce, Rockford, IL, USA). Membranes were blocked in TBST buffer with 5% goat serum for 1 hour at room temperature, incubated with the primary antibody then washed and incubated with the corresponding peroxidase-conjugated secondary antibody. Anti- α smooth muscle actin monoclonal antibody (Sigma, St. Louis, MO, USA) was used to detect expression of α smooth muscle actin. Antibodies against GAPDH (Sigma) were used for

detecting the protein loading control. Immune complexes were visualized using an ECL kit (Pierce, USA). The gray value of α -actin and GAPDH was analyzed with ImageJ image analysis software.

Enzyme linked immunosorbent assay (ELISA)

Cell culture supernatants were collected. Interleukin (IL)-6 and RANTES (Regulated on Activation, Normal T Expressed and Secreted) were quantified using ELISA kits (R&D, Minneapolis, MN, USA) according to the procedure recommended by the manufacturer. Obtained values were normalized to cell counts.

Statistical analysis

Data were expressed as mean $\pm$ standard error (SE) and analyzed by one-way analysis of variance (ANOVA) and *t*-test using SPSS 13.0 (SPSS Inc., USA). Differences were considered statistically significant at *P* < 0.05.

RESULTS

Establishment of rat asthma model

In the control group animals, the feeding, activity and respiratory rhythm looked normal with no signs of asthma. In the asthma group, there was evidence of shortness of breath, irregular respiratory rhythm, a slight tremor of the limbs, irritability, cough, camponotus, and a reduction in activity. At higher levels of allergens, the mouth and nose appeared slightly cyanotic. Slow response, erect hair, and poor color were also sometimes observed when allergens were inspired continuously. In addition, the thickness of both the airway wall and the smooth muscle layer normalized by airway basement perimeter was substantially greater in the chronic asthmatic rats than in the control rats (Figure 1).

Phenotype of control and asthmatic ASMCs

Under an inverted phase contrast microscope, individual smooth muscle cells appeared either with a spindle-shaped contractile phenotype, or with a proliferative/secretory phenotype (Figure 2A and 2B). Cells with the proliferative/secretory phenotype had a number of cellular processes, were rich in cytoplasm, of high-density (greater contrast under phase contrast microscopy), and had the nucleus in the center of the cell. At a low cell density, the control cells grew into an intertwined mesh pattern, whereas at high density, the cells organized into a spiral-shaped or fence-like pattern, with some cells overlapped forming the typical “peak-like” growth. Cells in the asthma group were larger than in the control group, and formed irregular growth patterns, with increased numbers of pseudopodia. Staining for α -actin positively identified smooth muscle cells in the cultures (Figure 2C and 2D).

Electron micrographs of the control group (Figure 3A) showed that the cells had a large area of cytoplasm that contained little rough endoplasmic reticulum, a small Golgi complex, few mitochondria, and extended few

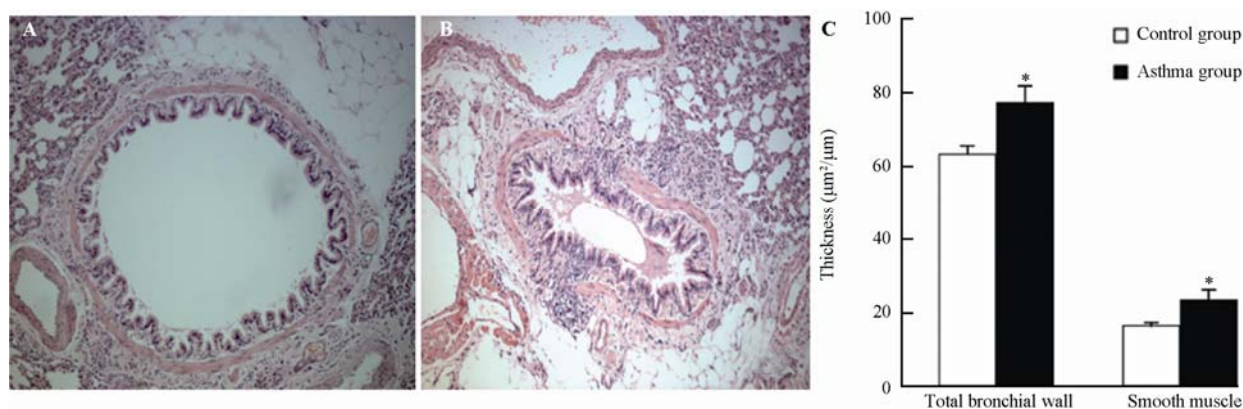


Figure 1. Hematoxylin and eosin staining of bronchial lung sections from SD rats (original magnification $\times 100$). The photomicrographs are representative of lungs removed from the normal control group (A) and OVA-challenged asthma group (B). In the asthma group, there was an apparent increase in the thickness of airway wall and smooth muscle layer. Results show the relative area of airway wall and airway smooth muscle based on the airway basement perimeter (C). $n=10$ rats; $*P<0.01$ versus control rats.

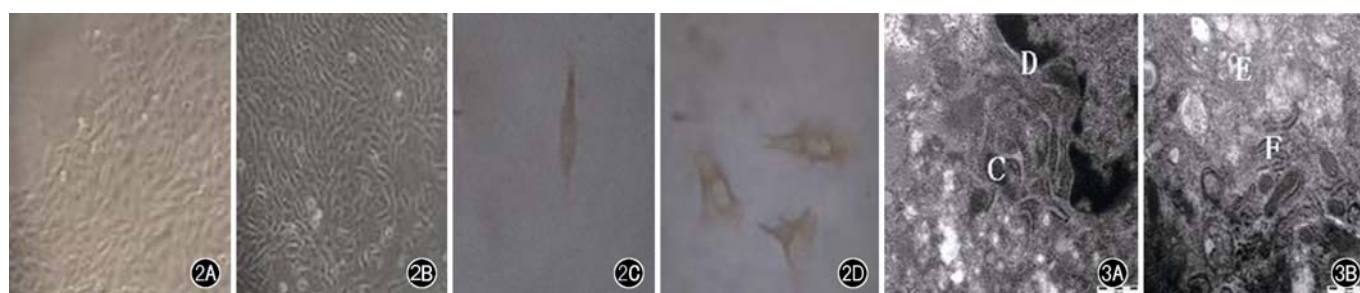


Figure 2. Morphology of ASMCS. Phase contrast microscopy (original magnification $\times 100$) of seven-day-old primary cultures of ASMCS from control rats (2A) and induced asthma rats (2B). Immunohistochemical staining for α -actin (brown) shows the different morphologies of cells from control rats (2C) and induced asthma rats (2D) (original magnification $\times 200$).

Figure 3. Ultrastructural comparison of cells from control and induced asthma rats (Original magnification $\times 39\,000$). 3A: Control group cells had less endoplasmic reticulum, Golgi, and mitochondria, and showed electron-dense cytoplasmic bodies (C), as well as an intact nuclear membrane (D). 3B: Asthma group cells had more Golgi (E), rough endoplasmic reticulum (F), mitochondria, and secretory vesicles.

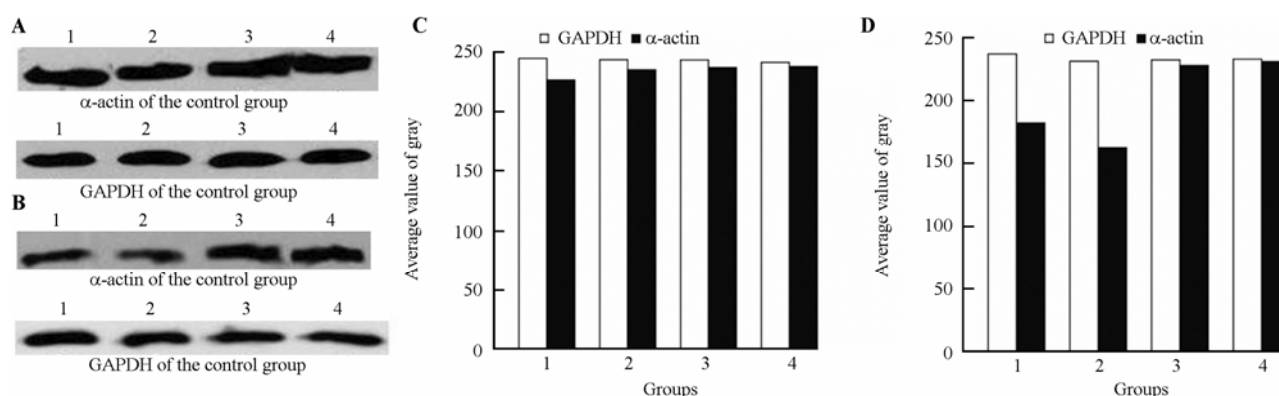


Figure 4. Variation in α -actin expression between treatment groups. A and B show bands from Western blotting for α -actin (top row) compared to GAPDH (bottom row), while C and D graph densitometric quantification of the bands. C: Data for control group (1: blank control group. 2: induced control group. 3: interventional control group I. 4: interventional control group II). D: Data for induced asthma cells (1: blank asthma group. 2: induced asthma group. 3: interventional asthma group I. 4: interventional asthma group II).

pseudopod-like membrane processes. The cells had a round nucleus with an intact nuclear membrane. In contrast, cells from the asthma group (Figure 3B) were rich in rough endoplasmic reticulum, Golgi complex, and mitochondria. The endoplasmic reticulum was tubular and various transport vesicles could be seen near the endoplasmic reticulum and under the plasma membrane. The nucleus had an irregular nuclear membrane, a larger

nucleolus, and had depressions in the outline of the nucleus.

Western blotting of α -actin

Figure 4 and Table show that the expression of α -actin in the control group did not differ significantly between the four treatment groups. In the asthma group the gray expression of α -actin was lower in the blank and induced

Table. Comparison of average density among different groups (mean $\pm$ SD, $n=4$)

Groups	α -Actin	GAPDH	α -Actin/GAPDH
Blank control	226.67 $\pm$ 19.37	244.72 $\pm$ 3.63	0.9257 $\pm$ 0.0697*
Induced control	235.16 $\pm$ 4.87	243.68 $\pm$ 4.20	0.9653 $\pm$ 0.0308*
Intervention control I	237.23 $\pm$ 13.6	243.44 $\pm$ 2.36	0.9742 $\pm$ 0.0478*
Intervention control II	238.03 $\pm$ 6.88	241.37 $\pm$ 3.63	0.9861 $\pm$ 0.0235*
Blank asthma	168.39 $\pm$ 14.5	233.28 $\pm$ 7.22	0.7198 $\pm$ 0.0394†‡
Induced asthma	165.70 $\pm$ 23.99	231.21 $\pm$ 9.41	0.7153 $\pm$ 0.0822†‡
Intervention asthma I	210.58 $\pm$ 36.45	232.33 $\pm$ 3.81	0.9815 $\pm$ 0.0533*
Intervention asthma II	231.01 $\pm$ 20.56	232.89 $\pm$ 2.05	0.9921 $\pm$ 0.0913*

* $P < 0.05$ compared with blank asthma group, † $P < 0.05$ compared with blank control group, ‡ $P < 0.05$ compared with intervention asthma I.

groups. Upon intervention with FPT III, either with or without the addition of the stimulant mixture, the α -actin level returned to the level of the control group.

IL-6 and RANTES secretion by ASMCs

Cell cultures from asthmatic rats secreted significantly more IL-6 than cell cultures from control rats (Figure 5, column 1 versus columns 5 and 6), and this increase was reversed by treatment with the p21Ras inhibitor FPT III (Figure 5, columns 7 and 8). FPT III also decreased IL-6 secretion in cell cultures from control rats (Figure 5, columns 3 and 4), but the decrease did not reach statistical significance. The effect of FPT III treatment was not dramatically affected by the addition of the stimulant mixture containing IL- β , TNF- α , and IFN- γ (Figure 5, columns 4 and 8). Likewise, the stimulant mixture had no obvious effect on IL-6 secretion in the cell cultures from the control rats (Figure 5, columns 2 and 6). However, it should be noted that there were non-significant trends for the stimulant mixture to increase IL-6 production.

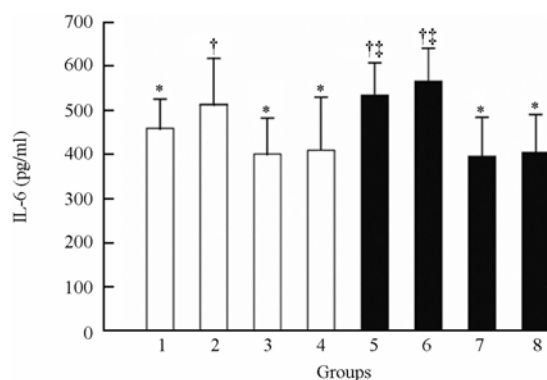


Figure 5. IL-6 secretion following asthma-induction, stimulation, and p21Ras inhibition (mean $\pm$ SE, $n=4$). Data from cells derived from control rats (white bars) are compared to that from asthmatic rats (black bars). 1: blank control group; 2: induced control group; 3: interventional control group I; 4: interventional control group II; 5: blank asthma group; 6: induced asthma group; 7: interventional asthma group I; 8: interventional asthma group II. * $P < 0.05$ compared with the blank asthma group, † $P < 0.05$ compared with the blank control group, ‡ $P < 0.05$ compared with interventional asthma group I.

A similar effect was observed with respect to the cytokine RANTES (Figure 6). Cell cultures from asthmatic rats secreted significantly more RANTES than cell cultures from control rats (Figure 6, column 1 versus columns 5

and 6), and this increase was reversed by treatment with FPT III (Figure 6, columns 7 and 8). FPT III had no effect on RANTES secretion in cell cultures from control rats (Figure 6, columns 3 and 4). Compared to IL-6 secretion, the effect of the stimulant mixture was more pronounced on RANTES secretion. In cell cultures from the control rats, the stimulant mixture significantly increased secretion of RANTES protein relative to the control blank group (Figure 6, column 2). In other experimental groups the stimulant mixture tended to increase the RANTES secretion, but did not do so to a significant level.

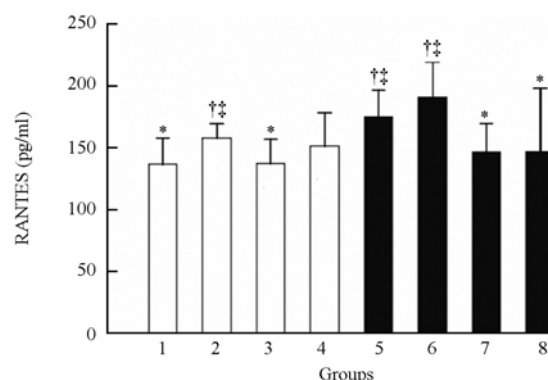


Figure 6. RANTES secretion following asthma-induction, stimulation, and p21Ras inhibition (mean $\pm$ SE, $n=4$). Data from cells derived from control rats (white bars) are compared to that from asthmatic rats (black bars). 1: blank control group; 2: induced control group; 3: interventional control group I; 4: interventional control group II; 5: blank asthma group; 6: induced asthma group; 7: interventional asthma group I; 8: interventional asthma group II. * $P < 0.05$ compared with the blank asthma group, † $P < 0.05$ compared with the blank control group, ‡ $P < 0.05$ compared with interventional asthma group I.

DISCUSSION

ASMCs are an important factor of airway physiology in asthma due to their ability to contract and control the diameter of the bronchi and bronchioles that it encircles. Asthma pathology might be influenced by the different phenotypes of ASMCs.¹² The mechanism of ASMC phenotype plasticity is still obscure.¹³ The initial discoveries of the p21Ras gene mainly focused on its potent carcinogenic properties,¹⁴ and the relationship between the ASMCs phenotype plasticity and p21Ras has not been reported until recently.

This study observed the ultrastructure of ASMCs by transmission electron microscopy and found that the ASMCs from the control group had a more slender profile, and had more dense bodies and dense plaques compared with the ASMCs from the asthma group. This meant there were more myofilaments in the normal ASMCs. We also found the cells from asthmatic rats had increased signs of secretory activity, such as increased rough endoplasmic reticulum, more mitochondria, more prominent Golgi apparatus, tubular and vesicle-like structures and secretory vesicles near the plasma membrane. In addition, the nuclei of the cells from control rats were round with nuclear membrane integrity,

whereas in ASMCs from asthmatic rats the nuclei had larger nucleoli, irregular nuclear membranes, more nuclear membrane recesses, and more nuclear chromatin aggregation. These signs suggested increased DNA synthesis in ASMCs from the asthma group. Taken together, the ultrastructural observations showed signs of increased protein production and secretory activity, as well as a change in cell shape in ASMCs from asthmatic rats.

In the past, evaluation of animal models of asthma was based on the clinical manifestation and pathological analysis. The results of this study showed that ASMCs exhibit differences in ultrastructure between normal rats and asthmatic rats. These ultrastructural changes agreed with the clinical and pathological changes seen during phenotype change. The ultrastructural data not only confirmed that a proliferative/secretory ASMC phenotype appeared in our asthma model, but also added new details about the phenotypic changes. These data will help us to evaluate the asthma model, and to identify details of the ASMC phenotype change.

FPT III was developed as an inhibitor of protein farnesyl-transferases. By inhibiting farnesylation, p21Ras is prevented from associating with the plasma membrane, breaking the signal transduction chain and preventing p21Ras-mediated effects. FPT III is extremely potent, and low doses can have a significant inhibiting effect. When the dose is greater than 100 $\mu\text{mol/L}$, FPT III is toxic to cells.¹⁵ Therefore, this experiment used a low dose of FPT III to specifically inhibit p21Ras protein function. Western blotting of α -actin allowed us to examine the phenotypic change in ASMCs. We found that FPT III treatment had no significant impact on α -actin expression in ASMCs from normal rats, indicating that these ASMCs were of the contractile phenotype. In this phenotype, p21Ras protein would be expected to have low activity and to be unphosphorylated, so there should be a less impact of FPT III on α -actin. However, it was found that α -actin levels were reduced in ASMCs from asthmatic rats (Figure 4). p21Ras protein would be expected to have been activated in asthma, inhibiting the α -actin gene promoter.¹⁶ Consequently, after the interference of p21Ras function by FPT III, the α -actin expression level of the cells from asthmatic rats increased (Figure 4). The ASMCs tended to have a more shrunken morphology, suggesting that p21Ras affected the ASMC phenotype, likely by regulating expression and distribution of α -actin.

The cytokine IL-6 is one of main regulators of inflammatory cells with a wide range of biological activities. ASMCs are an important source of IL-6. We found that IL-6 secretion levels were not significantly different between the control blank group and other groups, showing that p21Ras inhibition had little effect on normal cells. There was a significant difference between the asthma group and the control group,

indicating that p21Ras is involved in the activation of the abnormal secretion of IL-6. Increased secretion of IL-6 is known to occur when infectious factors invade the body, leading to expression of C-reactive protein which results in cell damage and further inflammation.¹⁷ This pathway could play an important role in the smooth muscle phenotype of the airway remodeling.^{18,19} Although the IL-6 levels in the induced asthma group increased, it was not significant relative to the blank asthma group; possibly due to the fact that the cells from the asthmatic rats had already been stimulated during the asthma-induction process to the point where no further stimulation was possible. The FPT III treatment to inhibit p21Ras activity significantly reduced IL-6 secretion in cells from asthmatic rats ($P < 0.05$). Based on these results, we concluded that p21Ras intervention can effectively reduce IL-6 secretion from asthmatic ASMCs, and may be able to reduce the pro-inflammatory effects resulting from the change of ASMCs to the proliferative/secretory phenotype.

ASMCs not only secrete inflammatory mediators, such as IL-6, but also secrete chemokines. RANTES (also known as CCL5) is a chemokine of the CC subfamily and is involved in regulating the body's immune response. This study showed that the ASMCs of the blank control group secreted more RANTES after induction with the stimulatory mixture. The expression of RANTES in the blank asthma group significantly increased compared with the blank control group, showing that the secretion from asthmatic rat ASMCs, proliferative/secretory phenotype, is greater than from the normal ASMCs, contractile phenotype. After intervention with FPT III, the secretion of RANTES in the asthma group significantly decreased ($P < 0.05$), showing that p21Ras is involved in RANTES secretion from asthmatic ASMCs. There was no significant difference in the level of RANTES secretion between the blank asthma group and induced asthma group. This result suggests that the normal ASMCs could change from synthesis phenotype to secretion phenotype when stimulated by the outside inflammatory factors. The synthesis and secretion of asthma ASMCs had no significant change when the p21Ras in asthmatic ASMCs had been activated.

The signaling pathway of p21Ras is a complex network that controls a wide range of cellular activities.²⁰⁻²² This study showed that p21Ras had an important influence on ASMC phenotype. Rats with induced asthma had ASMCs with a proliferative/synthetic phenotype which could be reverted toward a contractile phenotype when p21Ras was inhibited by treatment with FPT III. There was little effect of FPT III on ASMCs from normal rats, which were already a contractile phenotype. As a result, inhibiting p21Ras expression may effectively prevent the signal that promotes the proliferative/secretory phenotype transition of the cell, which may also inhibit expression of cytokines and inflammatory mediators.²³

In recent years, a number of specific and effective ways

to inhibit Ras have been developed for the treatment of tumors with abnormally activated p21Ras. Examples include the farnesyltransferase inhibitors (FTIs), such as FPT III, as well as receptor tyrosine kinase substrate and Src homology 2 area (SH2) or peptides of SH3 design. These inhibitors have achieved the desired results in animal models and pre-clinical experiments, and are currently in clinical trials.²⁴ Our research indicates that this technology could be transferred to the treatment of asthma and that p21Ras is a promising new target for the treatment of asthma.

Acknowledgements: We thank the Laboratory Center of Jinan University for providing electron microscopy and the team of Dr. LUO Shen-qiu in the Cell Biology Laboratory of Southern Medical University for the technical assistance in the course of this study.

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(Received April 27, 2010)

Edited by WANG Mou-yue and LIU Huan